

EXHIBIT A

Obtained from Trademark Electronic Search System (TESS)

<http://tess2.uspto.gov/bin/showfield?f=doc&state=4009:dn8j3v.2.1>

February 8, 2011

Word Mark	FELIDOVAC
Goods and Services	(EXPIRED) IC 005. US 018. G & S: FELINE INFECTIOUS ENTERITIS VACCINE
Mark Drawing Code	(1) TYPED DRAWING
Serial Number	72250345
Filing Date	July 15, 1966
Current Filing Basis	44E
Original Filing Basis	44E
Registration Number	0838226
Registration Date	November 7, 1967
Owner	(REGISTRANT) BEHRINGWERKE AKTIENGESELLSCHAFT CORPORATION FED REP GERMANY MARBURG (LAHN) FED REP GERMANY
Type of Mark	TRADEMARK
Register	PRINCIPAL
Affidavit Text	SECT 15.
Live/Dead Indicator	DEAD

EXHIBIT B

使用説明書

使用前に必ず本使用説明書を読み、注意事項を守って使用して下さい。

フェリドバック® PCR

猫ウイルス性鼻気管炎・猫カリシウイルス感染症・猫汎白血球減少症
混合（アルミニウムゲルアジュバント加）不活化ワクチン

〔製法及び性状〕

本剤は、猫ウイルス性鼻気管炎ウイルス 605 株、猫カリシウイルス 255 株、猫カリシウイルス 2024 株、猫汎白血球減少症ウイルス LV 株を猫腎由来株化 (CRFK) 細胞で増殖させ、得られたウイルス液にホルマリンを添加して不活化したものをそれぞれ混合し、アジュバントを加えたものである。静置すると沈殿を認めるが、振とうすれば帯赤色、やや不透明な懸濁液である。

〔成分及び分量〕

ワクチン 1mL 中

主剤	ネコ腎細胞培養ネコウイルス性鼻気管炎ウイルス 605 株 (不活化前ウイルス含有量)	10 ^{6.3} TCID ₅₀ 以上
	ネコ腎細胞培養ネコカリシウイルス 255 株 (不活化前ウイルス含有量)	10 ^{7.3} TCID ₅₀ 以上
	ネコ腎細胞培養ネコカリシウイルス 2024 株 (不活化前ウイルス含有量)	10 ^{7.3} TCID ₅₀ 以上
	ネコ腎細胞培養ネコ汎白血球減少症ウイルス LV 株 (不活化前ウイルス含有量)	3072HAU 以上
不活化剤	ホルマリン	0.25mg 以下
アジュバント	L80	15μg
	水酸化アルミニウム	2mg 以下
溶 剤	リン酸緩衝食塩液	残量

〔効能又は効果〕

猫ウイルス性鼻気管炎、猫カリシウイルス感染症、猫汎白血球減少症の予防。

〔用法及び用量〕

生後 8 週齢以降の猫 1 匹あたり 1mL ずつ、2～3 週間隔で 2 回皮下に接種する。

■ 使用上の注意 ■

〔一般的注意〕

- (1) 本剤は定められた用法・用量を厳守すること。
- (2) 本剤は効能、効果において定められた適応症の予防のみに使用すること。
- (3) 本剤は要指示医薬品であるので獣医師の処方せん・指示により使用すること。

〔使用者に対する注意〕

誤って人に注射した場合は、直ちに医師の診察を受けること。

〔対象動物に対する注意〕

1. 制限事項

- (1) 本剤の注射前には健康状態について検査し、異常を認めた場合は接種しないこと。
- (2) 対象猫が、次のいずれかに該当すると認められる場合には、健康状態及び体質等を考慮し、注射適否の判断を慎重に行うこと。
 - ・発熱又は下痢など臨床的異常が認められるもの。
 - ・疾病の治療を継続中又は治療後間がないもの。

・交配後まもないもの、分娩間際又は分娩直後のもの。

- ・妊娠中又は高齢のもの。
- ・重度の皮膚疾患が認められるもの。
- ・明らかな栄養障害があるもの。

(3)本剤を注射後、副反応(アナフィラキシー等)による事故を最小限にとどめるため、注射後しばらくは観察を続けること。帰宅させる場合は、なるべく安静に努めながら帰宅させ、当日は帰宅後も良く観察すること。

(4)注射当日から2～3日間は安静に努め、激しい運動、交配、入浴又はシャンプー等は避けること。

2. 副反応

(1)本剤の注射後、一過性の腫脹が認められる場合がある。

(2)本剤の注射直後に一過性の副反応(疼痛、食欲の不振、下痢又は嘔吐等)が認められる場合がある。

(3)過敏体質の猫では、まれにアレルギー反応、又はアナフィラキシー反応が起こることがある。アナフィラキシー反応は、本剤注射後30分くらいまでに見られる。

(4)猫ではワクチン注射により線維芽肉腫の発生率がわずかに高まるとの外国の報告がある。

(5)副反応が認められた場合は、速やかに獣医師の診察を受けるように指導するとともに、副反応に対しては適切な処置を行うこと。

3. 相互作用

(1)本剤には他の薬剤を加えて使用しないこと。

(2)本剤と他のワクチンとの同時注射は避けること。また、本剤注射前に他のワクチンを注射している場合には、生ワクチンにあっては1か月以上、不活化ワクチンにあっては1週間以上の間隔をあけること。なお、本剤注射後、他のワクチンを注射する場合は、1週間以上の間隔をあけること。

4. 適用上の注意

(1)移行抗体価の高い個体では、ワクチン効果が抑制されることがあるので、幼若な猫への注射は、移行抗体の消失する時期を考慮すること。

(2)注射器具は、滅菌されたものを使用すること。薬剤による消毒又は他の薬剤を使用した器具は、使用しないこと。なお、乾熱、高圧蒸気滅菌又は煮沸等を行った場合には、室温まで冷えたものをを用いること。

(3)本剤容器のゴム栓は、70%アルコール綿で消毒し、滅菌済みの注射針をゴム栓から刺し込み、注射筒内に吸引して使用すること。

(4)注射部位は、70%アルコール綿で消毒し、注射時に注射針が血管に入っていないことを確認してから注射すること。

(5)注射針は1頭ごとに取り替えること。

(6)開封して一度注射針を刺したワクチンは、速やかに使用し、使い残りのワクチンは、使用しないこと。

〔取扱上の注意〕

(1)アルミキャップでの切傷に注意すること。

(2)良く振り混ぜてから使用すること。

(3)外観及び内容に異常を認めたものは、使用しないこと。

(4)有効期限が過ぎたものは使用しないこと。

(5)ワクチン瓶は、破損する恐れがあるので、強い衝撃を与えないこと。

(6)使い残りのワクチン及び使用済みのワクチン瓶は、消毒又は滅菌後適切に処分すること。

〔保管上の注意〕

(1)小児の手の届かない所に保管すること。

(2)直射日光、高温又は凍結は品質に影響を与えるので、避けること。

〔貯法及び有効期間〕


1) 2～8℃

2) 有効期間は、製造後 30 か月。(最終有効年月は外箱に表示)

〔包装〕

1mL(1頭分)×10 バイアル

製造元 INTERVET INTERNATIONAL GmbH

輸入元  社団法人 北里研究所
東京都港区白金五丁目9番1号


販売元  明治製薬株式会社
東京都中央区京橋2-4-16

EXHIBIT C



Vaccine efficacy of a cell lysate with recombinant baculovirus-expressed feline infectious peritonitis (FIP) virus nucleocapsid protein against progression of FIP

Tsutomu Hohdatsu^{a,*}, Hiroshi Yamato^a, Tasuku Ohkawa^a,
Miyuki Kaneko^a, Kenji Motokawa^b, Hajime Kusahara^{a,b},
Takashi Kaneshima^a, Setsuo Arai^b, Hiroyuki Koyama^a

^a Department of Veterinary Infectious Diseases, School of Veterinary Medicine and Animal Sciences,
Kitasato University, Towada, Aomori 034, Japan

^b Research Center for Biologicals, The Kitasato Institute, Kitamoto, Saitama 364, Japan

Received 31 December 2002; received in revised form 27 August 2003; accepted 2 September 2003

Abstract

The Type II feline infectious peritonitis virus (FIPV) infection of feline macrophages is enhanced by a monoclonal antibody (MAb) to the S protein of FIPV. This antibody-dependent enhancement (ADE) activity increased with the MAb that showed a neutralizing activity with feline kidney cells, suggesting that there was a distinct correlation between ADE activity and the neutralizing activity. The close association between enhancing and neutralizing epitopes is an obstacle to developing a vaccine containing only neutralizing epitopes without enhancing epitopes. In this study, we immunized cats with cell lysate with recombinant baculovirus-expressed N protein of the Type I FIPV strain KU-2 with an adjuvant and investigated its preventive effect on the progression of FIP. Cats immunized with this vaccine produced antibodies against FIPV virion-derived N protein but did not produce virus-neutralizing antibodies. A delayed type hypersensitivity skin response to N protein was observed in these vaccinated cats, showing that cell mediated immunity against the FIPV antigen was induced. When these vaccinated cats were challenged with a high dose of heterologous FIPV, the survival rate was 75% (6/8), while the survival rate in the control group immunized with SF-9 cell-derived antigen was 12.5% (1/8). This study showed that immunization with the cell lysate with baculovirus-expressed N protein was effective in preventing the progression of FIP without inducing ADE of FIPV infection in cats. © 2003 Elsevier B.V. All rights reserved.

Keywords: Feline infectious peritonitis; Baculovirus; Vaccine; Nucleocapsid protein

* Corresponding author. Tel.: +81-176-23-4371; fax: +81-176-23-8703.
E-mail address: hohdatsu@vmas.kitasato-u.ac.jp (T. Hohdatsu).

1. Introduction

Feline infectious peritonitis (FIP) is a virus-induced chronically progressive, immunologically-mediated and usually fatal disease in domestic and wild *Felidae*. The causative agent of this disease is FIP virus (FIPV) which belongs to the family *Coronaviridae*. To prevent FIP, various vaccines such as virulence-attenuated live or inactivated FIPV vaccine have been investigated, but no vaccine has exhibited a sufficient effect and the vaccines rather enhanced the onset of FIP (Woods and Pedersen, 1979; Pedersen et al., 1981; Pedersen and Black, 1983; Pedersen et al., 1984; Barlough et al., 1984; Pedersen, 1987; Stoddart et al., 1988). Intraperitoneal inoculation of virulent FIPV induced more severe clinical signs in anti-FIPV antibody-positive kittens and kittens that received passive immunization with serum or purified IgG from antibody-positive cats than in antibody-negative kittens (Pedersen and Boyle, 1980; Weiss and Scott, 1981). The antibody-induced enhancement of FIPV infection is a serious obstacle to the prevention of FIP by vaccination.

Generally, macrophages play an important role in the non-specific defense against viral infections. However, it is also known that some viruses bound to antibodies invade macrophages via the Fc region of the antibody and the Fc gamma receptor (Fc γ R) of the macrophage, and eventually, the antibody leads to the enhancement of infection. This phenomenon is called antibody-dependent enhancement (ADE) of virus infection (Halstead and O'Rourke, 1977). Macrophages are known to be one of the target cells for FIPV and it has been reported that the ADE of FIPV infection is induced by the same mechanism (Hohdatsu et al., 1991a; Corapi et al., 1992; Olsen et al., 1992; Hohdatsu et al., 1998).

FIPV consists of three major proteins, nucleocapsid (N) protein, transmembrane (M) protein and peplomer (S) protein. Among these proteins, neutralizing and ADE epitopes are mainly present on the S protein (Hohdatsu et al., 1991a; Corapi et al., 1992; Olsen et al., 1992). We previously reported that in vitro FIPV infection of feline alveolar macrophages is enhanced by a monoclonal antibody (MAb) to the S protein of FIPV. This ADE activity increased with the MAb that showed a neutralizing activity with feline kidney cells, suggesting that there was a distinct correlation between ADE activity and the neutralizing activity (Corapi et al., 1992; Olsen et al., 1992; Hohdatsu et al., 1993). The close association between enhancing and neutralizing epitopes is an obstacle to developing a vaccine containing only neutralizing epitopes without enhancing epitopes. Although a recombinant vaccine has been prepared by inserting the gene encoding S protein into vaccinia virus, the vaccine did not prevent FIPV infection and it rather enhanced the onset of FIP (Vennema et al., 1990).

The effects of recombinant vaccines prepared by inserting the genes encoding FIPV M or N proteins into vaccinia virus (Vennema et al., 1991) or raccoon poxvirus (Wasmoen et al., 1995) and a DNA vaccine containing the *IL-12* gene with the M and N genes (Glansbeek et al., 2002) on FIPV infection have been investigated. Among these studies, only the vaccine using recombinant raccoon poxvirus expressing the N gene (rRCNV–FIPVN) reported by Wasmoen et al. (1995) was effective against a low-dose FIPV challenge. However, since they challenged feline enteric coronavirus (FECV) orally after immunization with rRCNV–FIPVN, their study did not show the effect of the vaccine alone. Moreover, this vaccine was a recombinant live vaccine and its field application requires the resolution of many problems including its safety.

A temperature-sensitive mutant strain of FIPV has been produced, and appears to be both safe and efficacious against a low dose homologous experimental challenge (Christianson et al., 1989; Gerber et al., 1990). However, there is still controversy over the safety and efficacy of this vaccine in that protection may depend on the strain and dose of the challenge virus (Scott et al., 1992; Scott et al., 1995; McArdle et al., 1995). The vaccinated cats showed ADE when challenged with a high dose of a heterologous virus strain (Scott et al., 1992; Scott et al., 1995). Therefore, no vaccine is sufficiently safe and effective against FIPV infection at present.

In this study, we immunized cats with the cell lysate with baculovirus-expressed N protein of the Type I FIPV strain KU-2 with an adjuvant and investigated its preventive effect on the onset of FIP. The vaccinated cats produced antibodies against N protein but did not produce virus-neutralizing antibodies. Cell mediated immunity is considered to play an important role in the prevention of the onset of FIP. A delayed type hypersensitivity (DTH) skin response to N protein was observed in these vaccinated cats. When the vaccinated cats were challenged with a high dose of heterologous FIPV, the onset of FIP was prevented without the induction of ADE and the survival rate was 75%.

2. Materials and methods

2.1. Cells and viruses

Spodoptera frugiperda (Sf)-9 cells were grown in monolayers at 27 °C in TC-100 medium containing 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin. *Felis catus* whole fetus (fcwf)-4 cells were cultured as monolayers at 37 °C in a humidified atmosphere of 5% CO₂ in Eagle's minimum essential medium containing 50% Leibovitz's L-15 medium, 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin. Type I FIPV strain KU-2 and Type II FIPV strain 79-1146 were grown in fcwf-4 cells at 37 °C. The FIPV KU-2 strain was isolated in our laboratory, and the FIPV 79-1146 strain was supplied by Dr. M.C. Horzinek of State University Utrecht, The Netherlands. A recombinant baculovirus expressing FIPV N protein was grown in SF-9 cells at 27 °C.

2.2. Cats

Anti-feline coronavirus (FCoV) antibody-negative specific pathogen-free cats aged 6–9 months were used. The cats were maintained in a temperature-controlled isolated facility.

2.3. Preparation of recombinant baculovirus-expressing the FIPV N protein

Genomic RNA was extracted from FIPV strain KU-2 infected culture fluid. The reverse transcription (RT) of genomic RNA and amplification of cDNA by polymerase chain reaction (PCR) were carried out using a standard technique with a DNA thermal cycler. RT-PCR primers were designed to cover the entire 1134 bp FIPV strain KU-2N gene (Accession no. AB086881). The PCR products were cloned into pFastBac1 plasmid. *Escherichia coli* DH10Bac strain was transfected with the recombinant plasmid DNA and cultured. The

white colonies grown were selected and the recombinant bacmid DNA was recovered. SF-9 cells were transfected with the recombinant bacmid DNA using cell fectin, and the culture supernatant was used in the experiment as recombinant baculovirus-expressed FIPV N protein.

2.4. Preparation of vaccine

SF-9 cells cultured for 2 days were inoculated with the recombinant baculovirus. After absorption for 1 h, serum-free TC-100 medium was added to the cells and the cells were cultured at 27 °C. After culture for 96 h, the infected cells were recovered and washed with PBS. One milliliter of RSB buffer containing 0.2% NP-40 (0.01 M NaCl, 0.0015 M MgCl₂, 0.01 M Tris-HCl, pH 7.4) was added to 1×10^7 cells and the cell suspension was kept at 4 °C for 30 min with occasional shaking. The cells were centrifuged at $800 \times g$ for 10 min. The precipitate was resuspended in PBS and used as recombinant N protein. Feline inactivated trivalent vaccine (Felidovac PCR; Intervet, The Netherlands), which is commercially available in Japan, was added to the recombinant N protein as an adjuvant. This feline inactivated trivalent vaccine contains 2% aluminum hydroxide gels and L80 as an adjuvant. The specificity of recombinant N protein and the amount of antigen were measured by enzyme-linked immunosorbent assay (ELISA) and Western immunoblotting using an anti-FIPV monoclonal antibody. ELISA and Western immunoblotting were performed by the method of Hohdatsu et al. (1991b).

SF-9 cell-derived antigen was prepared as the control antigen by NP-40 treatment of SF-9 cells on Day 4 of culture as in the recovery of recombinant N protein.

2.5. Purification of N protein from detergent-disrupted FIPV virions

The detergent-disrupted virions of FIPV strain 79-1146 purified by discontinuous sucrose density gradient ultracentrifugation were separated by SDS-PAGE. The position corresponding to the molecular weight of N protein was estimated from the positions of marker proteins, and the gel at the position was recovered. N protein was electrophoretically eluted from the recovered gel using protein elution apparatus (Maxyfield GP; Atto Corp., Japan). The specificity of N protein was investigated using MAbs recognizing FIPV S, M, and N proteins (Hohdatsu et al., 1991b). The electrophoretically purified N protein was used as an antigen to detect the antibodies in ELISA and the measurement of the DTH skin response.

2.6. Design of immunization/challenge procedure

To evaluate the efficacy of the recombinant N protein vaccine, the same vaccination/challenge experiment was repeated twice. Eight SPF cats aged 6 months and eight SPF cats aged 7–9 months were used in the first and second experiments, respectively. In both experiments, four cats were subcutaneously vaccinated three times with 3-week intervals. As a challenge control, four cats received a subcutaneous administration of the SF-9 cell-derived antigen, which was prepared as the recombinant N protein described above, with the adjuvant. Four weeks after the third vaccination, all cats were challenged oronasally with 10^5 TCID₅₀ FIPV strain 79-1146. These cats were observed daily for clinical disease signs,

and the body temperature and body weight were measured every 3 days. In the second experiment, oropharyngeal swabs were obtained every 3 days for virus isolation. Serum was collected every 6 days for the detection of anti-FIPV antibodies.

2.7. Serological assays

Antibody responses to FIPV nucleocapsid were detected by ELISA using N protein electrophoretically purified from detergent-disrupted FIPV virions. FIPV-neutralizing activity in heat-inactivated cat sera were determined as described previously using fcwf-4 cells in a 96-well microplate assay (Hohdatsu et al., 1992). The antibody titer was expressed as the reciprocal of the highest dilution of serum that completely inhibited a viral cytopathic effect.

2.8. DTH skin response

The cell-mediated immune response to the FIPV antigen was measured by intradermal skin testing using N protein electrophoretically purified from detergent-disrupted FIPV virions. The left lateral abdomen of the vaccinated cats was shaved and disinfected with 70% ethanol, and 0.1 ml of 100 µg/ml antigen or PBS was intradermally injected into the each skin site. The diameter of swelling at the injection site was measured 24, 48, 72, and 96 h after injection using a caliper.

3. Results

The specificity of the recombinant baculovirus-expressed FIPV N protein was investigated by ELISA and Western immunoblotting using MABs. In the ELISA, two-fold serial dilutions of the recombinant N protein were immobilized on 96-well flat bottom Microelisa plates and reacted with MAb F80-1 against S protein, MAb F18-2 against M protein, or MAb E22-2 against N protein. As shown in Fig. 1A, the recombinant N protein did not react with F80-1 or F18-2, and reacted only with E22-2, which recognizes N protein. Similarly, the recombinant N protein reacted only with E22-2 on Western immunoblotting and a specific band was detected at the position corresponding to 45 kDa. The amount of the recombinant N protein antigen per dose of the vaccine was measured by Western immunoblotting using MAb E22-2. The amount of the antigen detected as a positive band up to 16-fold dilution (16 U per dose) by Western immunoblotting was contained in one dose of the vaccine (Fig. 1B).

Humoral and cell-mediated immune responses to the FIPV antigens were investigated in the cats immunized with the recombinant N protein vaccine and the control cats. An increase in the ELISA value against FIPV virion-derived N protein was observed in Week 4 after the third immunization in all vaccinated cats in the first and second experiments (Fig. 2). However, neutralizing antibody titers against the homologous strain KU-2 and the challenge virus strain 79-1146 did not increase even after the third immunization, and the titer was 1:10 or lower in all vaccinated cats. In the control cats immunized with SF-9 cell-derived antigen, neither the ELISA value nor neutralizing antibody titer increased. Three weeks

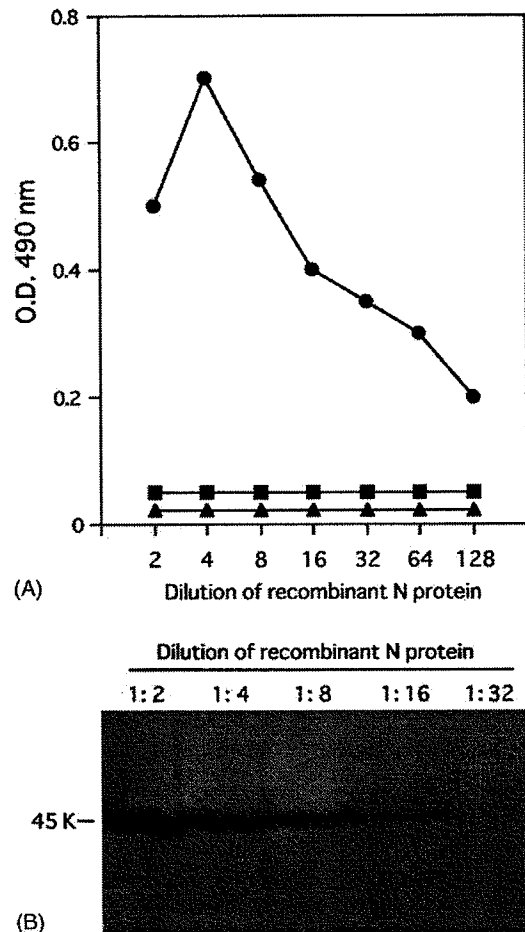


Fig. 1. Specificity of recombinant baculovirus-expressed FIPV N protein. (A) The specificity of recombinant N protein was investigated by ELISA using anti-FIPV MAbs. (●) Reactivity of MAb E22-2 recognizing FIPV N protein. (■) Reactivity of MAb F18-2 recognizing FIPV M protein. (▲) Reactivity of MAb F80-1 recognizing FIPV S protein. (B) Visualization of recombinant N protein on a Western blot after staining with MAb E22-2 recognizing FIPV N protein.

after the third immunization, 0.1 ml of FIPV virion-derived N protein was intracutaneously injected into the vaccinated cats, and the induction of DTH skin response to the FIPV antigen was investigated. In the four vaccinated animals in the first experiment, swelling at the N protein-injected skin site was observed after 24 h and the swelling persisted until 72 h. In these cats, no swelling was observed at the PBS-injected skin site. In the second experiment, swelling was also observed at the N protein-injected skin site in the four vaccinated cats. In cat no. O-3 in the second experiment, transient swelling was observed at the PBS-injected skin site after 72 h. Fig. 3 shows the diameters of the swelling measured using a caliper in the cats in the first and second experiments.

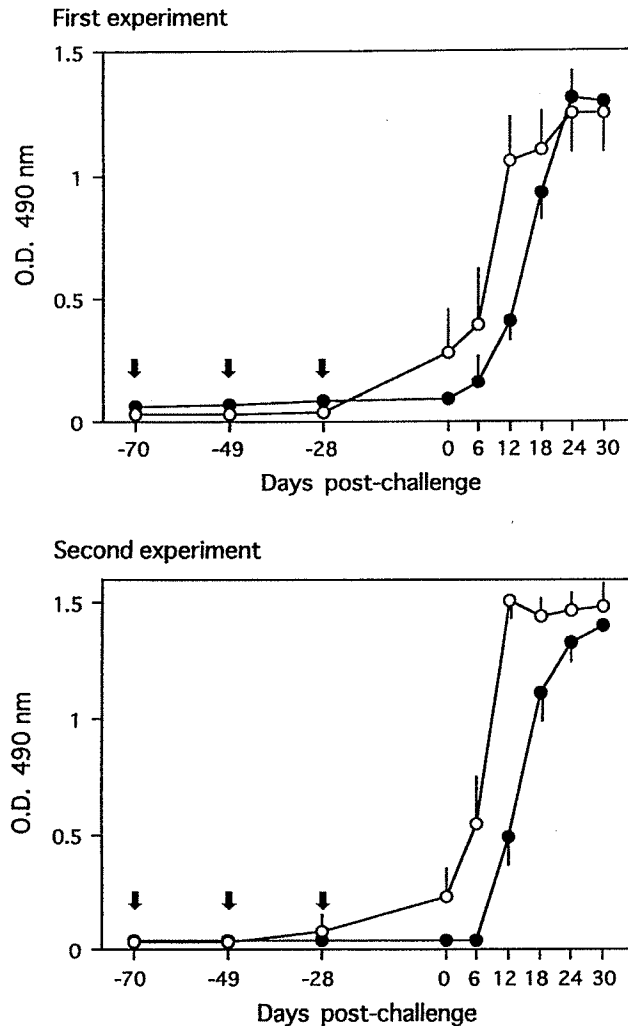


Fig. 2. Antibody levels in vaccinated and control cats before and after FIPV challenge. Sera collected at the indicated times were tested by ELISA using N protein electrophoretically purified from detergent-disrupted FIPV virions. Arrows indicate vaccine doses. (○) Mean \pm S.D. of ELISA OD-values in vaccinated cats. (●) Mean \pm S.D. of ELISA OD-values in control cats.

Four weeks after the third vaccination, all cats were challenged oronasally with 10^5 TCID₅₀ FIPV strain 79-1146. The antibody response to N protein, the neutralizing antibody titer against the challenge virus, changes in body weight, and the survival curves are shown in Table 1, Fig. 2, Figs. 4 and 5, respectively. In all vaccinated cats, the ELISA value against N protein began to increase on Day 6 after the challenge and the antibody responded earlier than that in the control cats (Fig. 2). The anti-challenge virus (strain 79-1146) neutralizing antibody production converted to positive on Day 12 after the challenge in the vaccination

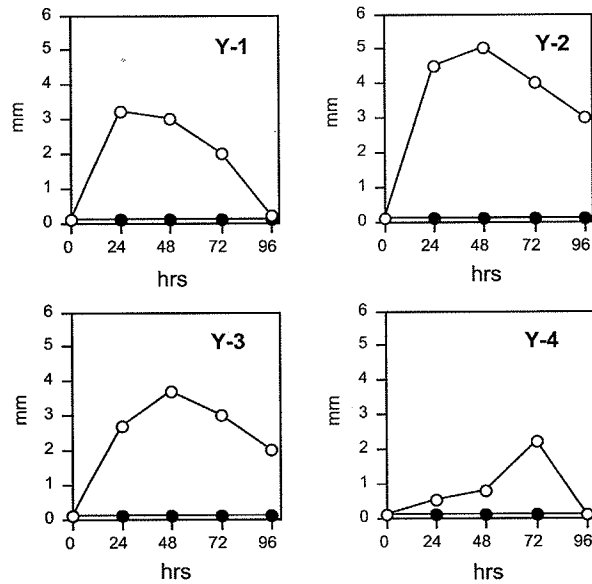
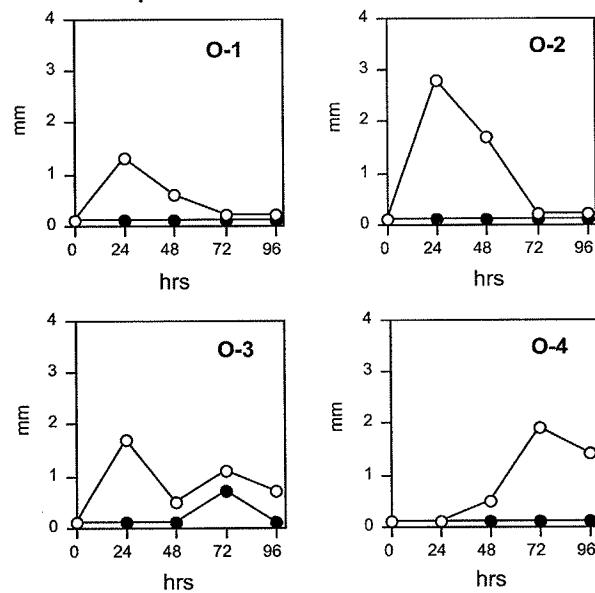
First experiment**Second experiment**

Fig. 3. Delayed-type hypersensitivity (DTH) skin response to FIPV N protein in vaccinated cats. Skin tests were performed by intra-dermally injecting either 0.1 ml of N protein electrophoretically purified from detergent-disrupted FIPV virions or 0.1 ml of PBS. The diameter of swelling at the injection site was measured 24, 48, 72, and 96 h after injection using a caliper. (○) N protein-injected skin site and (●) PBS-injected skin site.

Table 1
Neutralization antibody titer and onset of death following FIPV challenge

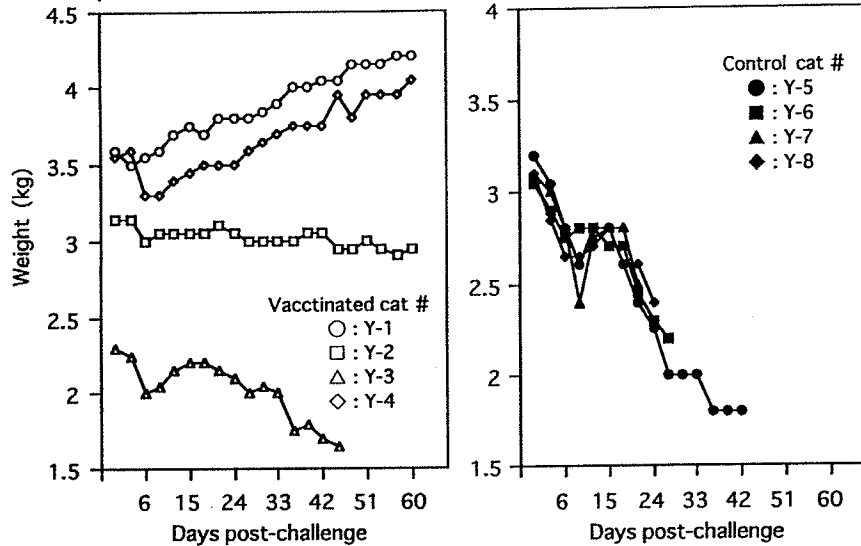
Group	Vaccine	Cat no.	Days post-challenge											Day of death
			0	12	18	19	23	26	29	31	44	48	60	
First exp.	Recombinant N protein	Y-1	<10	20	80								6400	Survival
		Y-2	<10	160	320								6400	Survival
		Y-3	<10	40	160							6400		48
		Y-4	<10	40	80								1600	Survival
	Controls (SF-9 cells)	Y-5	<10	80	320						6400			44
		Y-6	<10	80	160					1600				31
		Y-7	<10	80	160		800							23
		Y-8	<10	80	160				1600					26
Second exp.	Recombinant N protein	O-1	<10	40		200								19
		O-2	<10	20	80								6400	Survival
		O-3	<10	20	80								3200	Survival
		O-4	<10	10	40								3200	Survival
	Controls (SF-9 cells)	O-5	<10	10	40			400						29
		O-6	<10	40	160								3200	60
		O-7	<10	40	160								6400	Survival
		O-8	<10	40	80		200							23

and control groups, showing no significant difference between the two groups (Table 1). Samples obtained from oropharyngeal swabs collected every 3 days were inoculated with fcwf-4 cells to isolate the virus. The virus was isolated 3–9 days after the challenge from all cats in the vaccination and control groups, showing no significant difference in virus isolation. In the first experiment, the body weight gradually decreased and severe clinical disease developed in all cats in the control group, and the animals were euthanized 23–44 days after the FIPV challenge. The post-mortem examination findings were consistent with systemic pathological FIP disease. In contrast, in the vaccination group, cat Y-3 developed FIP but the other three animals developed no clinical signs of FIP during the 90-day observation period (Figs. 4 and 5). Similarly, in the second experiment, three of four animals in the control group developed systemic pathological FIP disease 23–60 days after the FIPV challenge and were euthanized. In the vaccination group, only cat O-1 developed FIP but the other three animals tolerated the challenge (Figs. 4 and 5). Combining the results of the first and second experiments, the survival rates were 75% (6/8) and 12.5% (1/8) for the immunized and control groups, respectively. These survival rates were analyzed using the χ^2 -test, and there was a significant difference ($P < 0.05$).

4. Discussion

This study confirmed that immunization of cats with baculovirus-expressed N protein was effective in preventing the progression of FIP following a high-dose (10^5 TCID₅₀) FIPV

First experiment



Second experiment

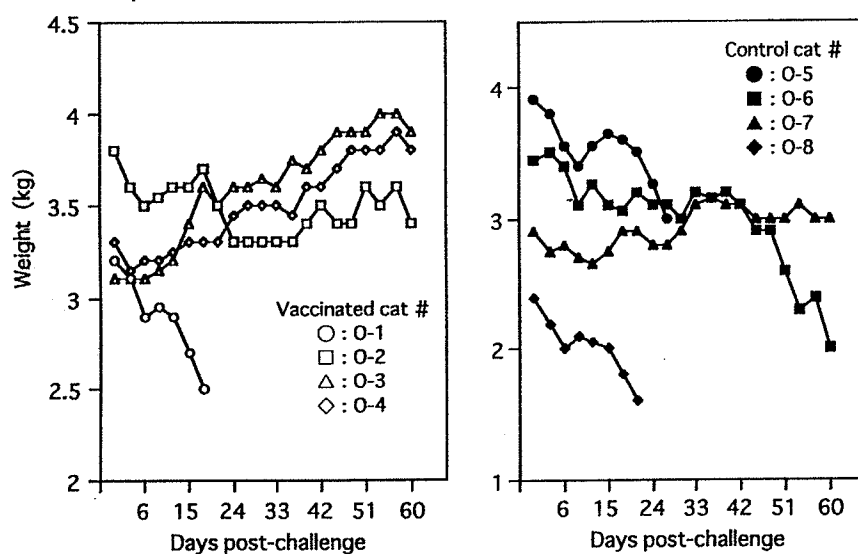
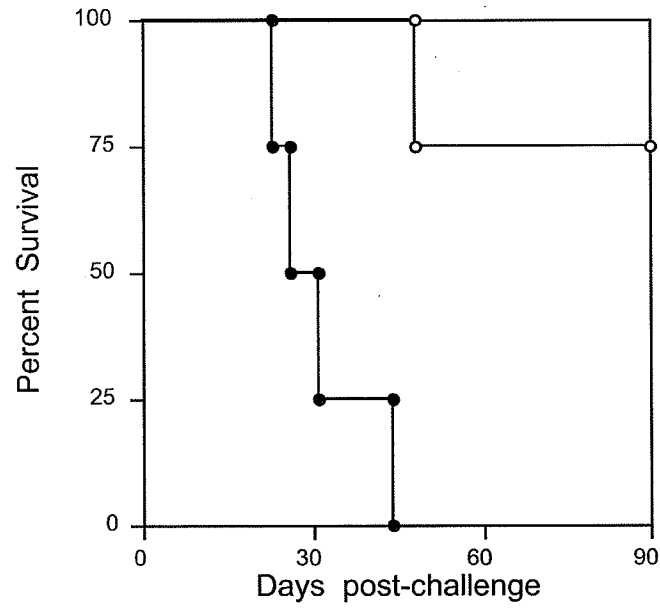


Fig. 4. Weight curves following FIPV challenge.

challenge. In cats immunized with this vaccine, the inhibition of FIP progression attained a survival rate of 75%. As described in the Introduction, Wasmoen et al. (1995) reported that in cats infected with FECV after immunization with recombinant racoon poxvirus expressing the *N* gene, the onset of FIP was 100% inhibited against a low-dose virulent

First experiment



Second experiment

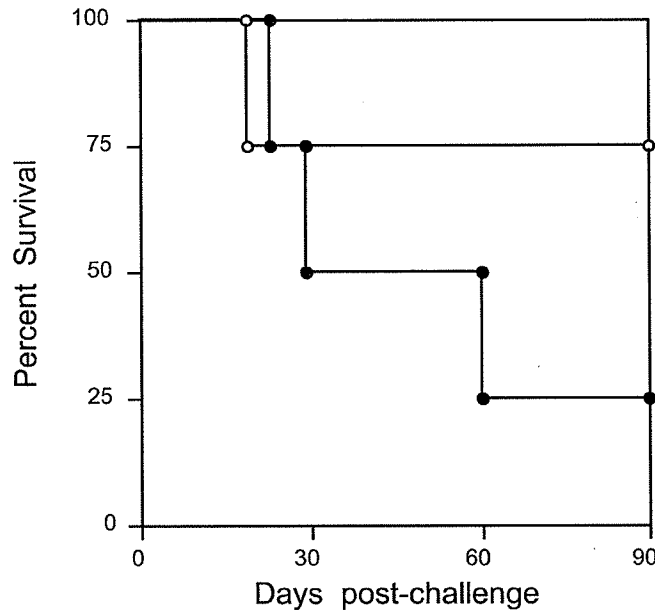


Fig. 5. Survival curves following FIPV challenge. (○) Vaccinates and (●) controls.

FIPV challenge (10^3 TCID₅₀). However, their study did not show the effect of the vaccine alone. Our study is the first to report that recombinant N protein alone attained a survival rate of 75% against a high-dose virulent FIPV challenge. Although we used Type I FIPV strain KU-2-derived recombinant N protein as the antigen, the vaccine also inhibited the onset of FIP following a challenge with Type II FIPV, 79-1146 strain. It is still unclear whether this immunization is effective against the same serotype FIPV challenge. Since the pathogenicity of oronasal challenge of KU-2 strain, which may be the natural route of infection, is weak, the vaccine effect on KU-2 strain could not be evaluated using the onset of FIP as the index in this study. Since Type I FIPV/FECV infection is dominant in the field (Hohdatsu et al., 1992), it is necessary to investigate the protective effects against Type I FIPV infection in the future. However, the amino acid sequence of N protein is conserved relatively well among FIPV strains (90% or higher homology; Motokawa et al., 1996) and infection with any type of FIPV may be controlled by use of this protein. No enhancing epitope present on N protein has been reported and no ADE was observed in this study, confirming that this protein is safe. In contrast, Gene analysis of S protein has shown that the amino acid sequences of Types I and II FIPV are very different (about 46% homology; Motokawa et al., 1996), suggesting that it is difficult to achieve an effect on infections with the two types of FIPV when S protein is selected for the vaccine antigen.

There was no difference in the virus isolation from oropharyngeal swabs collected after virulent FIPV challenge between the vaccinated and control cats in the second experiment, showing that FIPV infection was not prevented. However, virulent FIPV challenge-induced progression to FIP was prevented in the vaccinated cats. Cellular immunity is considered important for the prevention of progression to FIP (Pedersen, 1987). The vaccinated cats developed a DTH-skin response to N protein, suggesting that cell mediated immunity against the FIPV antigen was induced. Weiss and Cox (1988, 1989) reported that a strong DTH skin response to FIPV antigen was induced in cats that survived FIPV infection after FIPV challenge-exposure. Although the data are not shown, we observed no DTH response to virion-derived N protein in anti-FCoV antibody-negative SPF cats or cats that developed FIP, while cats that tolerated the FIPV challenge-exposure and FECV-infected cats exhibited a positive reaction. These findings strongly suggest that a DTH response to FIPV is associated with increased resistance to disease.

Generally, an inactivated vaccine induces humoral immunity because the vaccine is recognized as an exogenous protein, but the induction of cellular immunity is difficult. However, it has been found that antigens incorporated into immunostimulating complexes or encapsulated into certain liposomes can stimulate cytotoxic T lymphocyte (CTL) immunity (Takahashi et al., 1990; Reddy et al., 1992). In addition, Kovacsics-Bankowski et al. (1993) reported that soluble antigen conjugated to beads more efficiently induced CTL immunity than immunization with soluble antigen alone. It is considered that conversion of soluble to insoluble antigen by binding to beads increases phagocytosis of the antigen by antigen-presenting cells (APC), resulting in activation of MHC class I presenting pathway of exogenous proteins. The recombinant baculovirus-expressed FIPV N protein used in this study was insoluble antigen prepared as follows: recombinant baculovirus-infected SF9 cells were disrupted in RSB buffer containing 0.2% NP-40 and centrifuged at $800 \times g$ for 10 min, and the precipitate was resuspended in PBS. Use of insoluble N protein as the vaccine antigen may have induced cellular immunity including DTH response more efficiently. The

antigen prepared as described above may have contained baculovirus-derived antigens. It has recently been reported that baculovirus itself could induce non-specific anti-viral effects in mammals (Gronowski et al., 1999). Therefore, there is a possibility that baculovirus-derived antigen(s) has some synergetic effects on the successful protection.

The vaccine used in this study contained a high concentration of recombinant N protein (16 U on Western immunoblotting) and it prevented the onset of FIP without inducing ADE in a high-dose FIPV challenge. The vaccine may more effectively prevent the onset of FIP following a low-dose virus challenge close to spontaneous infection.

Acknowledgements

This work was supported in part by grants from the Ministry of Education, Science and Culture, Japan.

References

- Barlough, J.E., Stoddart, C.A., Sorresso, G.P., Jacobson, R.H., Scott, F.W., 1984. Experimental inoculation of cats with canine coronavirus and subsequent challenge with feline infectious peritonitis virus. *Lab. Anim. Sci.* 34, 592–597.
- Christianson, K.K., Ingersoll, J.D., Landon, R.M., Pfeiffer, N.E., Gerber, J.D., 1989. Characterization of a temperature-sensitive feline infectious peritonitis coronavirus. *Arch. Virol.* 109, 185–196.
- Corapi, W.V., Olsen, C.W., Scott, F.W., 1992. Monoclonal antibody analysis of neutralization and antibody-dependent enhancement of feline infectious peritonitis virus. *J. Virol.* 66, 6695–6705.
- Gerber, J.D., Ingersoll, J.D., Gast, A.M., Christianson, K.K., Selzer, N.L., Landon, R.M., Pfeiffer, N.E., Sharpee, R.L., Beckenhauer, W.H., 1990. Protection against feline infectious peritonitis by intranasal inoculation of a temperature-sensitive FIPV vaccine. *Vaccine* 8, 536–542.
- Glansbeek, H.L., Haagmans, B.L., te Lintelo, E.G., Egberink, H.F., Duquesne, V., Aubert, A., Horzinek, M.C., Rottier, P.J.M., 2002. Adverse effects of feline *IL-12* during DNA vaccination against feline infectious peritonitis virus. *J. Gen. Virol.* 83, 1–10.
- Gronowski, A.M., Hilbert, D.M., Sheehan, K.C.F., Garotta, G., Schreiber, R.D., 1999. Baculovirus stimulates antiviral effects in mammalian cells. *J. Virol.* 73, 9944–9951.
- Halstead, S.B., O'Rourke, E.J., 1977. Antibody-enhanced dengue virus infection in primate leukocytes. *Nature* 265, 739–741.
- Hohdatsu, T., Nakamura, M., Ishizuka, Y., Yamada, H., Koyama, H., 1991a. A study on the mechanism of antibody-dependent enhancement of feline infectious peritonitis virus infection in feline macrophages by monoclonal antibodies. *Arch. Virol.* 120, 207–217.
- Hohdatsu, T., Okada, S., Ishizuka, Y., Yamada, H., Koyama, H., 1992. The prevalence of types I and II feline coronavirus infections in cats. *J. Vet. Med. Sci.* 54, 557–562.
- Hohdatsu, T., Sasamoto, T., Okada, S., Koyama, H., 1991b. Antigenic analysis of feline coronaviruses with monoclonal antibodies (MAbs): preparation of MAbs which discriminate between FIPV strain 79-1146 and FECV strain 79-1683. *Vet. Microbiol.* 28, 13–24.
- Hohdatsu, T., Yamada, H., Ishizuka, Y., Koyama, H., 1993. Enhancement and neutralization of feline infectious peritonitis virus infection in feline macrophages by neutralizing monoclonal antibodies recognizing different epitopes. *Microbiol. Immunol.* 37, 499–504.
- Hohdatsu, T., Yamada, M., Tominaga, R., Makino, K., Kida, K., Koyama, H., 1998. Antibody-dependent enhancement of feline infectious peritonitis virus infection in feline alveolar macrophages and human monocyte cell line U937 by serum of cats experimentally or naturally infected with feline coronavirus. *J. Vet. Med. Sci.* 60, 49–55.

- Kovacsovics-Bankowski, M., Clark, K., Benacerraf, B., Rock, K.L., 1993. Efficient major histocompatibility complex class I presentation of exogenous antigen upon phagocytosis by macrophages. *Proc. Natl. Acad. Sci. U.S.A.* 90, 4942–4946.
- McArdle, F., Tennant, B., Bennett, M., Kelly, D.F., Gaskell, C.J., Gaskell, R.M., 1995. Independent evaluation of a modified live FIPV vaccine under experimental conditions. *Feline Pract.* 23, 67–72.
- Motokawa, K., Hohdatsu, T., Hashimoto, H., Koyama, H., 1996. Comparison of the amino acid sequence and phylogenetic analysis of the peplomer, integral membrane and nucleocapsid proteins of feline, canine and porcine coronaviruses. *Microbiol. Immunol.* 40, 425–433.
- Olsen, C.W., Corapi, W.V., Ngichabe, C.K., Baines, J.D., Scott, F.W., 1992. Monoclonal antibodies to the spike protein of feline infectious peritonitis virus mediate antibody-dependent enhancement of infection of feline macrophages. *J. Virol.* 66, 956–965.
- Pedersen, N.C., 1987. Virologic and immunologic aspects of feline infectious peritonitis virus infection. *Adv. Exp. Med. Biol.* 218, 529–550.
- Pedersen, N.C., Black, W.B., 1983. Attempted immunization of cats against feline infectious peritonitis, using avirulent live virus or sublethal amounts of virulent virus. *Am. J. Vet. Res.* 44, 229–234.
- Pedersen, N.C., Boyle, J.F., 1980. Immunologic phenomena in the effusive form of feline infectious peritonitis. *Am. J. Vet. Res.* 41, 868–876.
- Pedersen, N.C., Boyle, J.F., Floyd, K., Fudge, A., Barker, J., 1981. An enteric coronavirus infection of cats and its relationship to feline infectious peritonitis. *Am. J. Vet. Res.* 42, 368–377.
- Pedersen, N.C., Everman, J.F., McKeirnan, A.J., Ott, R.L., 1984. Pathogenicity studies of feline coronavirus isolates 79-1146 and 79-1683. *Am. J. Vet. Res.* 45, 2580–2585.
- Reddy, R., Zhou, F., Nair, S., Huang, L., Rouse, B.T., 1992. In vivo cytotoxic T lymphocyte induction with soluble proteins administered in liposomes. *J. Immunol.* 148, 1585–1589.
- Stoddart, C.A., Barlough, J.E., Baldwin, C.A., Scott, F.W., 1988. Attempted immunisation of cats against feline infectious peritonitis using canine coronavirus. *Res. Vet. Sci.* 45, 383–388.
- Scott, F.W., Corapi, W.V., Olsen, C.W., 1992. Evaluation of the safety and efficacy of Primucell-FIP vaccine. *Feline Health Top.* 7, 6–8.
- Scott, F.W., Corapi, W.V., Olsen, C.W., 1995. Independent evaluation of a modified live FIPV vaccine under experimental conditions. *Feline Pract.* 23, 74–76.
- Takahashi, H., Takeshita, T., Morein, B., Putney, S., Germain, R.N., Berzofsky, J.A., 1990. Induction of CD8⁺ cytotoxic T cells by immunization with purified HIV-1 envelope protein in ISCOMs. *Nature* 26, 873–875.
- Vennema, H., de Groot, R.J., Harbour, D.A., Dalderup, M., Gruffydd Jones, T., Horzinek, M.C., Spaan, W.J., 1990. Early death after feline infectious peritonitis virus challenge due to recombinant vaccinia virus immunization. *J. Virol.* 64, 1407–1409.
- Vennema, H., de Groot, R.J., Harbour, D.A., Horzinek, M.C., Spaan, W.J.M., 1991. Primary structure of the membrane and nucleocapsid protein genes of feline infectious peritonitis virus and immunogenicity of recombinant vaccinia viruses in kittens. *Virology* 181, 327–335.
- Wasmoen, T.L., Kadakia, N.P., Unfer, R.C., Fickbohm, B.L., Cook, C.P., Chu, H.-J., Acree, W.M., 1995. Protection of cats from infectious peritonitis by vaccination with a recombinant raccoon poxvirus expressing the nucleocapsid gene of feline infectious peritonitis virus. *Adv. Exp. Med. Biol.* 280, 221–228.
- Weiss, R.C., Cox, N.R., 1988. Delayed-type hypersensitivity skin response associated with feline infectious peritonitis in two cats. *Res. Vet. Sci.* 44, 396–398.
- Weiss, R.C., Cox, N.R., 1989. Evaluation of immunity to feline infectious peritonitis in cats with cutaneous viral-induced delayed hypersensitivity. *Vet. Immunol. Immunopathol.* 21, 293–309.
- Weiss, R.C., Scott, F.W., 1981. Antibody-mediated enhancement of disease in feline infectious peritonitis: comparisons with dengue hemorrhagic fever. *Comp. Immunol. Microbiol. Infect. Dis.* 4, 175–189.
- Woods, R.D., Pedersen, N.C., 1979. Cross-protection studies between feline infectious peritonitis and porcine transmissible gastroenteritis viruses. *Vet. Microbiol.* 4, 11–16.

EXHIBIT D

Table 1

1 dose (1 mL) Felidovac PCR contains:

Feline rhinotracheitis virus strain 605, inactivated	at least $10^{6.3}$ TCID ₅₀ *
Feline calicivirus strain 255, inactivated	at least $10^{7.3}$ TCID ₅₀
Feline calicivirus strain 2024, inactivated	at least $10^{7.3}$ TCID ₅₀
Feline panleukopenia virus strain LV, inactivated	at least 3072 HAU**
Aluminum hydroxide	less than 2 mg
Adjuvant L80	15 µg
Formaldehyde	less than 0.25 mg

*: 50% Tissue culture infectious dose

**: Haemoagglutinating units